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Morphogenetic Movements and Assembly of Strobilae into Zooidal Systems in Early Colony Development of the Compound Ascidian *Polyclinum planum*

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Abstract. Large-scale developmental processes, such as the morphogenetic movement of constructional sub-units within colonies of modular organisms, require self-assembling mechanisms. The compound ascidian *Polyclinum planum* is a modular organism. In the present study, colonies of *P. planum* were subjected to light-shock, and the development of the released tadpole larvae was followed in the laboratory from settlement, through the formation of oozooids and, finally, small colonies. Oozooids strobilated in 40 to 78 days. The number of post-abdominal strobilae produced per oozooid depended on post-abdomen length, but the timing of strobilation did not. Thoracic and abdominal remnants of *P. planum* parent zooids regressed completely; only the post-abdominal strobilae regenerated to form new zooids. Regenerating strobilae moved through the tunic into the region once occupied by the parent zooid, and formed a zooidal system around a pocket-like common cloacal cavity. Expansion of the thoraxes of regenerating zooids within the young colony produced the cylindrical to capitate shape of small, single-system colonies. The observed non-regeneration of the thoracic and abdominal remnants of *P. planum* differs markedly from their reported fates in other polyclinid ascidians. These observations shed light on the "rules of assembly" that govern formation of young *P. planum* colonies.

Introduction

Self-assembly mechanisms proceed without the direct control of the modular cell's DNA, usually occurring outside the cell and often at levels higher than that of the cell (Bonner, 1974; Adair, 1988; Edelman, 1988; Vreeland and Laetsch, 1988). Thus, development on an even broader scale must rely heavily upon supra-cellular activity.

Modular organisms demonstrate such large-scale organizational activities in their development and growth (Harper *et al.*, 1986). They grow through the iteration of functional units (modules), and in colony-forming species, the arrangement of modules in relation to each other determines colony shape and organization.

Compound ascidian tunicates are modular organisms. The diverse ways in which ascidian blastozooids (their modules) arise has been reviewed by Berrill (1935, 1951); Brien (1948, 1958); Nakauchi and Kawamura (1966); Nakauchi (1982); and Monniot *et al.* (1991).

Oozooid development, strobilation, and colony organization in ascidians of the family Polyclinidae have received considerable attention (Brien, 1924, 1925, 1936; Scott, 1952; Trason, 1957; Nakauchi, 1966, 1970, 1974, 1977, 1979, 1980a, b, 1981, 1982, 1986, 1987; Freeman, 1971; Nakauchi and Kawamura, 1974, 1978). These studies show that zooid production in polyclinid ascidians is by strobilation of either the abdomen or post-abdomen, or of both the abdomen and the post-abdomen. Descriptions of bud fates and system formation in this family have consistently shown that all buds—thoracic, abdominal, and post-abdominal—regenerate, with the thoracic remnant of the parent zooid usually regenerating to become the center of a new system of zooids (Brien 1936; Nakauchi 1966, 1970, 1977, 1979, 1981, 1986, 1987; Freeman 1971; Nakauchi and Kawamura 1974, 1978).

In the present paper, I use direct observation and time-lapse videomicroscopy to describe the strobilation of oo-

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zooids and blastozooids and the self-assembling movement and organization of consequent zooids into systems in the compound ascidian *Polyclinum planum*.

Materials and Methods

Early stages of colony morphogenesis in *Polyclinum planum*, from settled larvae through the formation of systems of zooids, were studied in culture at the Long Marine Laboratory, Santa Cruz, California. Larvae were obtained from colonies collected intertidally at Hopkins Marine Station, Pacific Grove, California. Larval release was induced by holding colonies in running seawater in the dark for at least 48 h, and then subjecting them to bright incandescent illumination (3.5×10^{20} to 5.5×10^{20} photons $cm^{-2}\ s^{-1}$). Zooids released their larvae 1–30 min after the initiation of the light shock. The tadpole larvae emerged from the common cloacal openings and swam directly toward the brightest light source, where they were easily collected and transferred to drops of water on 75 × 50 mm glass slides. They settled within 30 min. Cohorts of larvae were collected in this way in February and November 1990, and in April 1991.

Developing oozooids, and the colonies they subsequently produced, were maintained in 0.2 μm-filtered, aerated seawater, in closed, plastic 5-l containers. The containers were held at ambient laboratory seawater temperature (about 14°C; Fig. 1) by partial immersion in a running-seawater table. At least twice a week, when the water in the containers was changed, cultures were fed Liquifry marine food (concentration: 2 drops per liter) and unicellular algae: *Dunaliella* and *Isochrysis* (100 ml of each alga; approximate cell densities of 10,000 to 15,000 cells ml^{-1}). Liquifry is a commercial food for filter-feeding invertebrates in marine aquaria; its main ingredients are dextrin, pea flour, whole egg, yeast, spinach: 3.5% protein, 1.6% fat, 1% fiber. Liquifry was used successfully by Boyd *et al.* (1986) to rear laboratory-cultured Monterey *Botryllus schosseri*.

The post-settlement growth and development of each *Polyclinum planum* oozooid or colony was monitored weekly. The maximum lengths of every zooid's thorax, abdomen, and post-abdomen, the longest visible blood vessel in the tunic, and the total length of each oozooid or colony, including its tunic, was measured. These body parts were observed with an inverted microscope through the glass slide on which the cultures were growing.

Time-lapse videomicroscopy was used to augment the observations described above, and to record the pattern of zooid strobilation, the regeneration of strobilae, and the organization of multiple-zooid systems. A Panasonic PV-604 video camera was fitted to a dissection microscope, and the camera's pre-programmed time-lapse feature taped these events for one second of every minute. Strobilation of three zooids, the regeneration of about 15 strobilae, and the organization of two multiple-zooid systems were recorded in this manner.

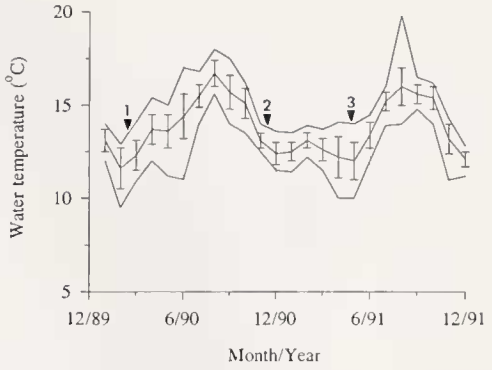

Figure 1. High, low, and mean monthly seawater temperatures (±SD) in seawater tables of the Long Marine Laboratory, Santa Cruz, California, from January 1990 through December 1991 (data provided courtesy of J. S. Pearse). Settlement dates of cohorts of *Polyclinum planum* followed during this study are indicated by arrows: 1 = 14 Feb 1990; 2 = 7 Nov 1990; 3 = 26 Apr 1991.

Results

Laboratory cultures of *Polyclinum planum* larvae, released after light shock, settled on glass slides on 14 February 1990 (n = 20 larvae), 7 November 1990 (n = 70), and 26 April 1991 (n = 32). These cohorts were maintained in culture until 27 November 1990, 3 June 1991, and 19 August 1991, respectively. The zooids showed evidence of feeding as soon as their oral and atrial apertures opened; *i.e.*, food was clearly visible in the gut, and fecal pellets were constantly produced.

Polyclinum planum oozooids strobilated on average 55.1 days after larval settlement (SD = 7.4 days; n = 92 oozooids). The earliest strobilation occurred in 40 days, and the latest at 78 days. Oozooids produced the body regions typical of all polyclinid ascidians: thorax, abdomen, and post-abdomen, as well as at least one test vessel that extended into the tunic from near the heart (Fig. 2).

The initiation of zooidal strobilation was signalled by the atrophy of the test vessels and by the regression of the thorax and abdomen. The oozooid's thorax soon separated from its abdomen at the esophagus. Next, the abdomen separated from the post-abdomen, and the former zooid was now divided into isolated thoracic, abdominal, and post-abdominal remnants. The isolated post-abdomen then produced one or more strobilae, the number depending on total post-abdominal length (Fig. 3). The lack of significance between the lengths of post-abdomens

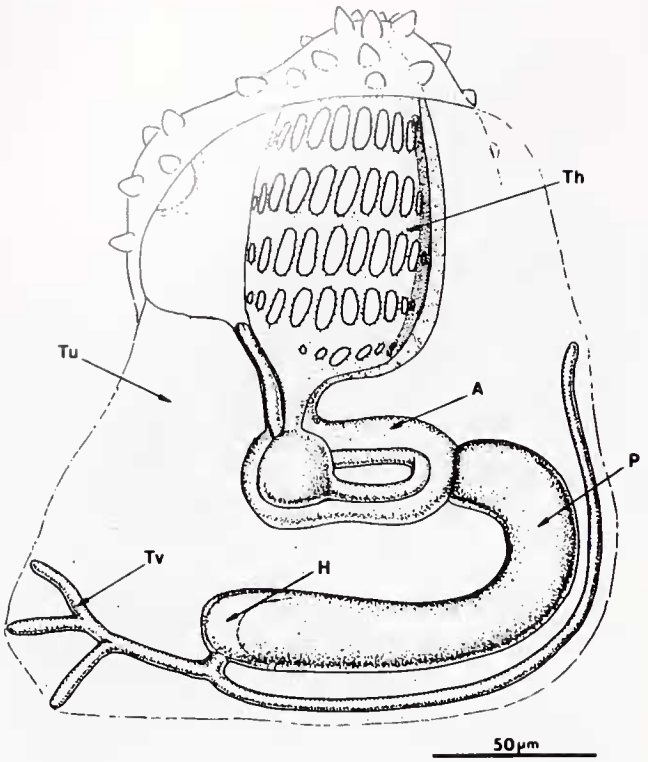

Figure 2. Fully developed oozooid of *Polyclinum planum* as viewed through the slide to which it is attached (A—abdomen; P—post-abdomen; H—heart; Th—thorax; Tv—test vessel; Tu—tunic). Tuberculate region at the top is the surface of the tunic around the oozooid's oral and cloacal apertures.

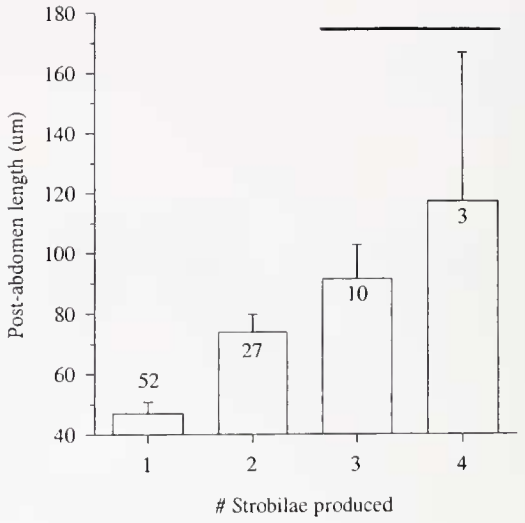

Figure 3. Mean post-abdomen lengths for groups of zooids producing 1 (n = 52 zooids), 2 (n = 27), 3 (n = 10), or 4 (n = 3) post-abdominal strobilae. Error bars are standard deviations. A Tukey multiple comparisons test revealed significant differences between post-abdomen lengths of zooids producing 1, 2, and 3 strobilae, but not between those producing 3 or 4 strobilae, as indicated by the Tukey-bar on the figure.

giving rise to three strobilae and those giving rise to four may be attributed to the small number of zooids (n = 3) that produced four post-abdominal strobilae. Although the number of strobilae produced was a function of post-abdomen length, the timing of strobilation was not (Fig. 4). Post-abdominal strobilae were pinched off sequentially, beginning "proximally," at the end formerly next to the abdomen, and proceeding "distally" toward the end farthest from the abdomen (Fig. 5).

Post-abdominal strobilae regenerated into whole blastozooids, while the thoracic and abdominal remnants regressed completely (Fig. 6). These regressing elements became granular, possibly supplying nutrients to the regenerating strobilae. (The regressed thorax precluded feeding at this [illegible]. All of the post-abdominal strobilae showed polarized development. That is, in each new strobila, a new thorax and abdomen formed at the end closest to the former abdomen.

Time-lapse recordings revealed the pattern of development in the regenerating strobilae. First, the strobilae produced a clear bulge at the end of the strobila formerly proximal to the abdomen (Fig. 5E–G; Fig. 6A). This clear bulge was the new developing thorax. At the same time, a smaller clear area—the heart—appeared at the end of the strobila opposite that of the new thorax. At this point, a strobila consisted of a post-abdomen and a thorax. Fi-

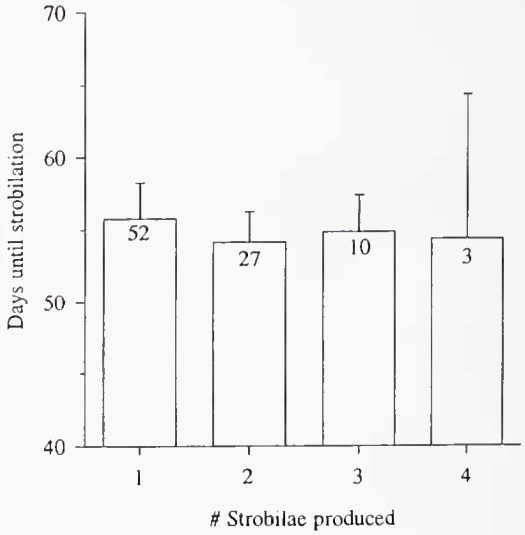

Figure 4. Time from larval settlement to strobilation of the oozooid, comparing oozooids producing 1 (n = 52 zooids), 2 (n = 27), 3 (n = 10), or 4 (n = 3) post-abdominal strobilae. Error bars are standard deviations. One-way ANOVA of the four groups of zooids indicated no significant differences between the four groups ($F = 0.30 < 2.76 = F_{0.05(3,60)}$; $P > 0.5$).

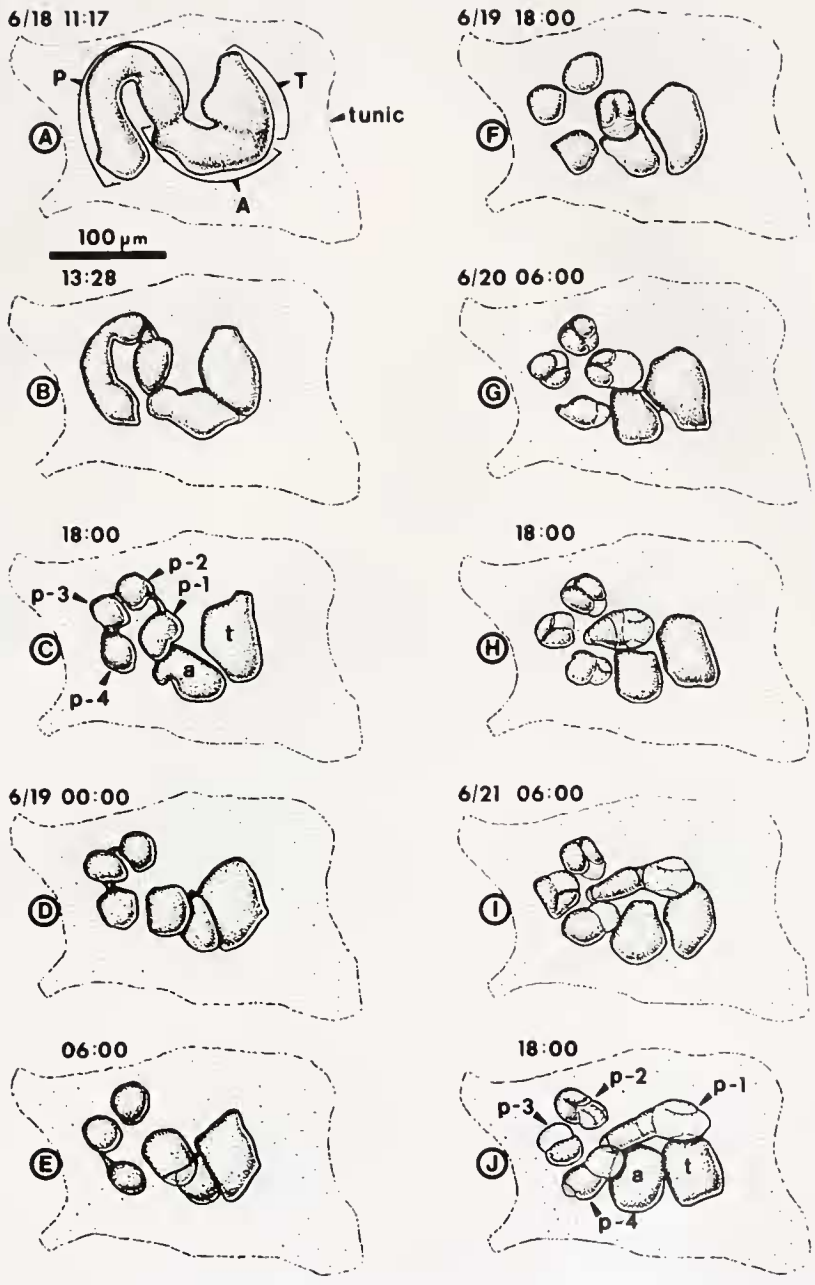

Figure 5. Pattern of strobilation of a *Polyclinum planum* oozooid, drawn from time-lapse video record (T—thorax; A—abdomen; P—post-abdomen; t—thoracic remnant; a—abdominal remnant; p-1 to p-4—post-abdominal strobilae). Read down columns. Figures A to J.

nally, an abdomen developed between the post-abdomen and the thorax. Each of the three body regions then increased in size, and were continuing to grow as the taping sessions ended.

Growth of the three body regions of the new blastozooid was accompanied by the movement of the entire bud through the colony's tunic, with the new thorax always in the lead. Rhythmic contractions of the post-abdomen, and then of the abdomen, occurred during these movements. Thoraxes, however, did not exhibit similar contractions as they expanded. Time-lapse video recordings showed that the mean contraction rate of post-abdomens was 42 min (pooled mean; SD = 15 min), and for the abdomens, 35.5 min (SD = 14 min). These "developmental contractions" occurred over a much longer period than either the mean heart rate of these zooids (x = 54

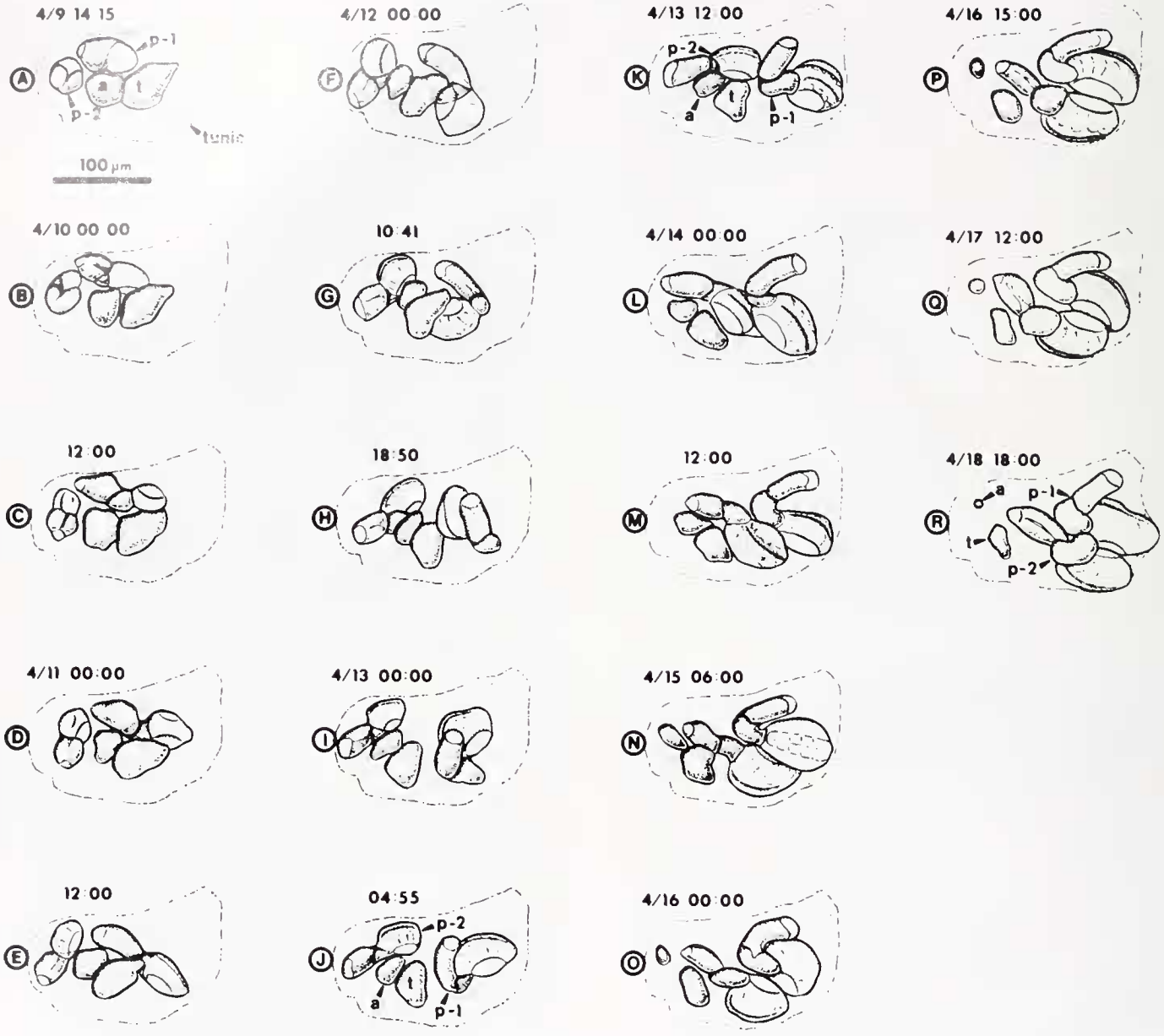

Figure 6. Two regenerating post-abdominal strobilae in the laboratory, drawn from time-lapse video record (t—thoracic remnant; a—abdominal remnant; p-1, p-2—regenerating post-abdominal strobilae). Note the decrease in size of thoracic and abdominal remnants as the post-abdominal strobilae regenerate. Read down columns, Figures A to R.

beats/min, SD = 2 beats/min), or the interval between the reversal of blood flow through the zooid's body, which occurred about every 2 min.

Regenerative buds always moved toward the region previously occupied by the thorax of the parent zooid, displacing the remaining parent zooid's thoracic and abdominal remnants (Fig. 6). Once in place, the new blastozooids arranged themselves into a zooidal system. The thoraxes of new zooids rotated until their endostyles lay farthest from each other, and their atrial (cloacal) languets lay closest. Once their rotation was completed, the zooids' cloacal (atrial) apertures emptied into the new system's pocket-like common cloacal cavity.

The aggregation of thoraxes, and their subsequent growth and expansion, swelled the thoracic region of the new colony's single system of blastozooids. This swelling gave the colony a somewhat cylindrical to capitate shape, with its one common cloacal cavity and aperture at the

top (Fig. 7). The base of the colony now formed the beginning of the peduncle that characterizes larger, multisystem colonies in this species.

Discussion

From these results, we can formulate at least tentative "rules of assembly" by which modules are formed and by which systems of zooids are organized during early colony development in *Polyclinum planum*. These rules are listed and discussed below.

First, post-abdomen length determines the number of strobilae produced per bout of strobilation (see Fig. 3). It may well be that post-abdomen growth is regulated by nutrition, as previously discussed by Freeman (1971), and that better nutrition will result in the production of more strobilae and, therefore, faster growth of the colony.

The initiation of strobilation appears to be internally regulated. The timing of strobilation of zooids did not differ significantly between zooids producing 1, 2, 3, or 4 post-abdominal strobilae (see Fig. 4). These results cast doubt on Brien's (1968) suggestion that lengthening of the post-abdomen leads to impaired blood circulation in it, and that this circulatory congestion triggers strobilation. Freeman (1971), on the other hand, reported that increased *thorax* length is related to strobilation. Freeman's siamese twin experiments (1971), in which a single zooid had both an older and a younger thorax, showed that the younger thorax determines the initiation of strobilation. A similar thorax or entire-zooid age-size effect may also determine the onset of strobilation in *Polyclinum planum*.

Polyclinum planum's pattern of strobilation resembles that of other polyclinids (Nakauchi, 1982). And the timing of strobilation in *P. planum* is close to that of the only congener studied so far: *P. aurantium* (40–70 days; Nakauchi, 1981).

Second, only post-abdominal strobilae regenerate; the thoracic and abdominal remnants completely regress. The fates of the isolated thoracic and abdominal elements of iterating *Polyclinum planum* zooids do not conform to those of other polyclinids. In other polyclinids, the thoracic and abdominal elements can also regenerate into new zooids. My results with *P. planum* show that the production of solely post-abdominal zooids occurred consistently in all cohorts, at different times of the year, and independently of post-abdomen length. But whether this strobilation pattern also occurs in the field, or is only an artifact is still unclear.

Reviewing other species, Nakauchi (1981) suggests that, under normal conditions, the polyclinid thoracic remnant persists and regenerates an abdomen and post-abdomen, but that the thorax and even the abdomen regress during "survival budding" (his term) under adverse conditions.

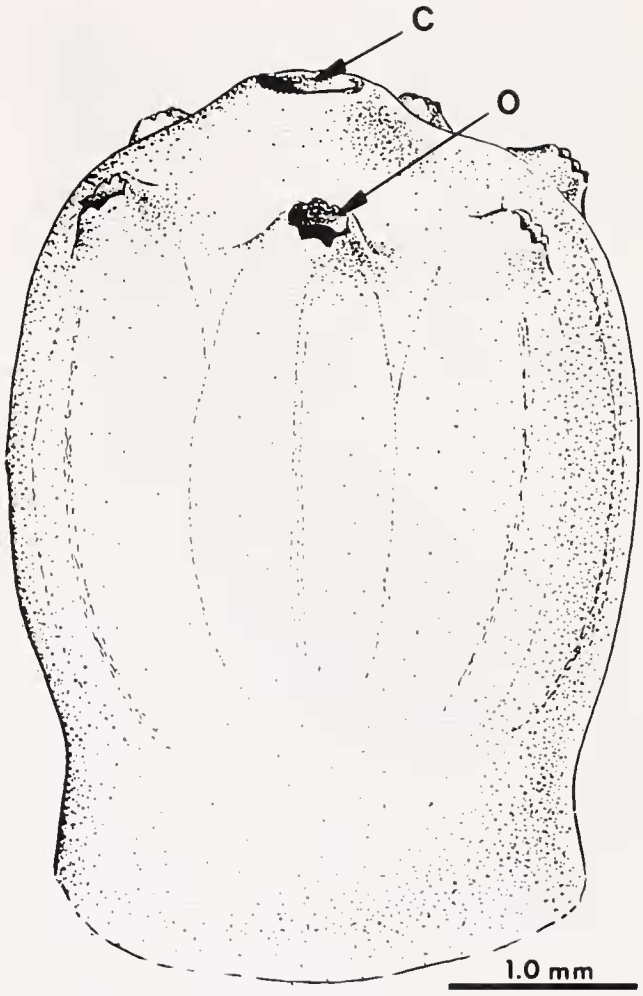

Figure 7. A young single-system colony of *Polyclinum planum* comprised of six zooids (C—common cloacal opening; O—oral aperture). Thoraxes of blastozooids are visible through the tunic. Blastozooids are much larger than oozooids (see Fig. 2) in this species.

Could my cultures have been under stress? Possibly, but zooids that can produce multiple post-abdominal buds certainly have enough energy stores to support the regeneration of their thoracic and abdominal fragments, if those fragments have the ability to regenerate at all. In *Polyclinum planum*, evidently, only post-abdominal fragments have this ability. On the other hand, laboratory culture and an artificial diet can produce stress resulting in abnormal growth.

If the strobila fates of *Polyclinum planum* can be further substantiated by rearing specimens in the field (which has not been attempted), then the pattern of strobilation reported here may warrant the addition of a new strobilation type to Nakauchi's (1982) inventory.

Third, all regenerating post-abdominal strobilae exhibit identical polarity in regeneration. The thorax, and then

the abdomen, each develop at the proximal end, and a new post-abdomen and heart develop at the distal end. This phenomenon facilitates the organization of an entire zooidal system from the regenerating strobilae, since strobilae tend to be oriented in the same direction as they are produced.

Fourth, regenerating strobilae move through the tunic as they develop, so that their thoraxes eventually lie in the space left by the regressed thorax of the parent zooid (see Fig. 6). This orderly arrangement is consistent with that reported by Nakauchi and Kawamura (1974, 1978) for regenerating strobilae of the polyclinid *Aplidium multiplicatum*. They found that regenerating strobilae move within the tunic, typically to a site either currently or previously occupied by a grown zooid or by a regenerating thoracic fragment. They proposed that grown zooids produce some attractant substance that organizes the movements of regenerating strobilae. Identifying any such attractant and its source would help us understand the control of self-assembly at levels higher than that of the individual module. But, of course, several other mechanisms, even pressure-differentials within the tunic or varying polymerization of polysaccharides in newer tunic, might also be hypothesized as controlling movement at this level.

Time-lapse video recordings of regenerating strobilae showed that their post-abdomens and abdomens had cyclic contractions as they moved through the tunic. These developmental contractions occurred over fairly long periods ($\bar{x}$ = 35 min for abdomens; $\bar{x}$ = 42 min for post-abdomens). The contractions are probably not driven by blood-flow reversals, or by the heart rate itself, because those phenomena operate within much shorter time frames. The causes and effects of these developmental contractions and the ultrastructural changes they generate within strobilae are, as yet, unstudied.

Fifth, system formation in *Polyclinum planum* commences as the thoraxes of regenerating strobilae aggregate in the region once occupied by the parent zooid's thorax. Thoraxes of newly regenerated blastozooids rotate until their endostyles lie on the rim of the circular system, and their future cloacal apertures point toward the center of the new system.

Sixth, as zooids reorient themselves this way, each zooid opens its oral (incurrent) and cloacal (excurrent) apertures. Each zooid maintains its own small oral siphon and aperture, which opens directly to the surface of the colony, while its cloacal aperture opens into a pocket-like common cloacal cavity that receives water from all zooids in the system. The system's common cloacal cavity opens to the surface of the colony as a rather large common cloacal aperture, slightly raised on a siphon.

Seventh, since several regenerating strobilae may result from the post-abdominal fragmentation of a single parent zooid, regenerating blastozooids must necessarily crowd into a limited space as they move toward the colony's surface. In the smallest colonies, this crowding, followed by the expansion of thoraxes and the production of tunic material by new zooids, enlarges the whole colony and generates the usual capitate shape. It is not yet clear that all regenerating *Polyclinum planum* zooids successfully reach the surface of the colony and join a system, especially in larger, multi-system colonies; some may well be crowded out and die.

In very young colonies, with few zooids or with only one or two post-abdominal strobilae, crowding does not tend to be a factor, nor do such colonies (*e.g.*, Fig. 6) assume the upright posture typical of the species, until more zooids are produced.

The events of module development and zooid regeneration are certainly under genetic control, but the forces governing system formation and whole colony morphogenesis remain problematic. How might genetic coding within the cells of a zooid extend its influence to the spatial arrangement of systems in which a zooid is only a modular component? Perhaps Nakauchi and Kawamura's (1978) "attractants"—substances produced by the parent zooid that affect movement of regenerating strobilae—hold a clue. System formation, including the molding of a common cloacal cavity and aperture and the differentiation of the whole colony into a basal peduncle and a lofted, zooid-bearing region, are important morphogenetic events. Genetic explanations provide for the production of the materials involved in these processes, but other contributing factors—physical ones like the effects simply of crowding zooids, and environmental ones like seasonal fluctuations in availability of food or nearness of other colonies—just as surely play their roles in guiding or molding these larger events in the self-assembly of the colony.

Although these tentative "rules of assembly" describe the production of strobilae and the formation of zooidal systems, still higher-order rules most certainly govern processes such as differentiation of an older *Polyclinum planum* colony into a peduncle and a zooid-bearing lobe, and influence morphogenetic changes in the zooid-bearing lobe as it increases in size and changes from a semispherical to a discoid shape—the shape by which *P. planum* is most often recognized in the field.

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